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Candida glabrata PDR1, a Transcriptional Regulator of a Pleiotropic Drug Resistance Network, Mediates Azole Resistance in Clinical Isolates and Petite Mutants

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***Candida glabrata*, a yeast with intrinsically low susceptibility to azoles, frequently develops increased azole resistance during prolonged treatment. Transposon mutagenesis revealed that disruption of Cg*PDR1* resulted in an 8- to 16-fold increase in fluconazole susceptibility of *C. glabrata*. Cg*PDR1* is a homolog of *Saccharomyces cerevisiae PDR1*, which encodes a transcriptional regulator of multidrug transporters. Northern blot analyses indicated that Cg*PDR1* regulated both constitutive and drug-induced expression of Cg*CDR1*, a multidrug transporter gene. In agreement with the Northern analysis, the Cg*pdr1* mutant had increased rhodamine accumulation, in contrast to the decreased accumulation in the Cg*PDR1*-overexpressing strain. Northern analyses also indicated the importance of Cg*PDR1* in fluconazole resistance arising during therapy. Two clinically resistant isolates had higher expression of Cg*PDR1* and Cg*CDR1* compared to their paired susceptible isolates. Integrative transformation of Cg*PDR1* from the two resistant isolates converted the Cg*pdr1* mutant into azole-resistant strains with upregulated Cg*PDR1* expression. Two different amino acid substitutions, W297S in one isolate and F575L in the other, accounted for the upregulated Cg*PDR1* expression and the resistance. Finally, Cg*PDR1* was shown to be required for the azole resistance due to mitochondrial deficiency. Thus, Cg*PDR1* encodes a transcriptional regulator of a pleiotropic drug resistance network and contributes to the azole resistance of clinical isolates and petite mutants.**

Candida glabrata has emerged as a common cause of bloodstream and mucosal infections in many countries, and it exhibits intrinsically low susceptibility to fluconazole (29, 35). This species is naturally about eightfold more resistant to fluconazole than *C. albicans* and easily develops further fluconazole resistance following prolonged therapy of patients with fluconazole (2, 30).

Azole antifungals target cytochrome P-450-dependent C14 lanosterol demethylase encoded by *ERG11* and interfere with ergosterol biosynthesis (17, 23, 43). Inhibition of ergosterol biosynthesis leads to the depletion of ergosterol, the major component of the fungal plasma membrane, and possibly accumulation of toxic sterol intermediates. The major mechanisms of azole resistance described to date have been primarily based on studies done in *C. albicans* and *Saccharomyces cerevisiae* and include overexpression of C14 lanosterol demethylase (13), alteration of the azole-binding site of C14 lanosterol demethylase (19, 22, 37), mutation in other ergosterol biosynthetic genes (18), and increased drug efflux. Increased drug efflux in *Candida* species is mainly due to increased expression of ATP-binding cassette (ABC) and major facilitator superfamily transporters (25, 28, 31, 34, 40). In addition to studies of the transporter and ergosterol biosynthetic genes at a transcriptional level, there also have been studies of posttranscriptional events in the genes of *C. glabrata*. Fluconazole treatment was shown to increase the presence of CgErg11p and CgCdr1p in the plasma membrane, and activity of CgCdr1p was modulated by phosphorylation (27, 39).

There are two lines of evidences which suggest that Cg*PDR1* is a transcriptional regulator of ABC transporters and plays a central role in fluconazole resistance acquired by *C. glabrata* during azole therapy or through mitochondrial dysfunction. High-frequency acquired resistance (HFAR) to fluconazole has been reported in *C. glabrata* and *S. cerevisiae* in the laboratory setting (4, 5, 7, 16, 33, 44), and overexpression of fluconazole-effluxing ABC transporters in the petite mutants has been documented in both *S. cerevisiae* and *C. glabrata* (33, 44). Two transcriptional factors, Pdr1p and Pdr3p, are known to regulate the pleiotropic drug resistance (PDR) network in *S. cerevisiae* (1, 9, 15). To date, *PDR3* but not *PDR1* has been shown to be essential for HFAR in *S. cerevisiae* (12, 44). However, the homolog of *PDR1* but not *PDR3* was annotated in the recently published *C. glabrata* genome sequences from the Pasteur Institute (11). The other line of evidence comes from Vermitsky and Edlind (38), who reported that expression of Cg*PDR1* was increased in one of seven *C. glabrata* mutants selected in vitro for fluconazole resistance. The strain with increased Cg*PDR1* expression differed from the parental Pdr1p by a single deduced amino acid, P927L, in the activation domain, raising the question of whether this mutation had caused the resistance.

We report here the role of Cg*PDR1* in fluconazole resistance using several approaches. We studied Cg*pdr1* mutants obtained by transposon mutagenesis (36) and by targeted gene disruption, including Cg*pdr1* mutants in a petite background. We also sequenced Cg*PDR1* and its 5′ flanking region in clinical isolates obtained before and after the appearance of fluconazole

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TABLE 1. Strains used in this study

Strain	Parental strain	Genotype and/or description	Reference or source
Candida glabrata			
38a		Clinical isolate	25
NCCLS84		Wild type	ATCC 90030
84u	NCCLS84	*ura3*	14
84870	84u	Cg*cdr1*::*URA3 pdh1*Δ::*ura3*	14
Cg12581		Clinical isolate, pair 1	2
Cg13928		Clinical resistant isolate, pair1	2
Cg1660		Clinical isolate, pair 4	2
Cg4672		Clinical resistant isolate, pair 4	2
Cg1660u	Cg1660	*ura3*	36
CgTn173S	Cg1660u	*ura3* Cg*pdr1*::Tn*5*<Cm URA3>	This study
CgB4	84u	*ura3* Cg*pdr1*::Tn*5*<Cm URA3>	This study
CgB4u	CgB4	*ura3* Cg*pdr1*::Tn*5*<Cm *ura3*>	This study
Cg173Cu	CgB4	*ura3*Cg*PDR1–13928*[a]	This study
Cg173C	Cg173Cu	*URA3* Cg*PDR1–13928 URA3* revertant of Cg173Cu	This study
CgB4u/pCgACU	CgB4u	*ura3* Cg*pdr1*::Tn5<Cm ura3> URA3	This study
CgB4u/pCgPDR1	CgB4u	*ura3* Cg*pdr1*::Tn5<Cm ura3> URA3 *ADH1*$_p$-Cg*PDR1*$_{38a}$-*ADH1*$_T$	This study
CgB4Ca$_{12581}$	CgB4	*ura3* Cg*PDR1–12581*[a]	This study
CgB4Cb$_{12581}$	CgB4	*ura3* Cg*PDR1–12581*[a]	This study
CgB4Ca$_{13928}$	CgB4	*ura3* Cg*PDR1–13928*[a]	This study
CgB4Cb$_{13928}$	CgB4	*ura3* Cg*PDR1–13928*[a]	This study
CgB4Ca$_{1660}$	CgB4	*ura3* Cg*PDR1–1660*[a]	This study
CgB4Cb$_{1660}$	CgB4	*ura3* Cg*PDR1–1660*[a]	This study
CgB4Ca$_{4672}$	CgB4	*ura3* Cg*PDR1–4672*[a]	This study
CgB4Cb$_{4672}$	CgB4	*ura3* Cg*PDR1–4672*[a]	This study
84p1	NCCLS84	Mitochondrion-deficient mutant	This study
84p2	NCCLS84	Mitochondrion-deficient mutant	This study
84p3	NCCLS84	Mitochondrion-deficient mutant	This study
CgB4p3	CgB4	Mitochondrion-deficient mutant	This study
CgB4p4	CgB4	Mitochondrion-deficient mutant	This study
CgB4p5	CgB4	Mitochondrion-deficient mutant	This study
Saccharomyces cerevisiae			
BY4741		MATa *his3Δ1 leu2Δ0 metΔ0 ura3Δ0*	3
4381	BY4741	MATa *his3Δ1 leu2Δ0 metΔ0 ura3Δ0 pdr1*	41
4381/pH 392	4381	MATa *his3Δ1 leu2Δ0 metΔ0 ura3Δ0 pdr1 URA3*	This study
4381/pHCgPDR1	4381	MATa *his3Δ1 leu2Δ0 metΔ0 ura3Δ0 pdr1 URA3* ADH1$_p$-Cg*PDR1*$_{38a}$-ADH1$_T$	This study

[a] Complementation by integrative transformation.

resistance (2). Sequence analysis and gene substitution experiments found that azole resistance and Cg*PDR1* expression were determined by single amino acid differences in the Cg*PDR1* open reading frame (ORF). The results indicate the central role of Cg*PDR1* in fluconazole resistance arising both during clinical use and through mitochondrial dysfunction.

MATERIALS AND METHODS

Strains and culture conditions. Plasmids were maintained in *Escherichia coli* XL1-Blue (Stratagene, La Jolla, CA), Top 10 (Invitrogen, Carlsbad, CA), or Top 10F′ (Invitrogen) host cells grown in 50 μg/ml ampicillin or 12.5 μg/ml chloramphenicol. EC100D *pir*$^+$ and EC100D *pir*-116 (Epicenter, Madison, WI) were used as hosts for the EZ::TN transposon (Tn) (Epicenter) which relies on the R6K origin for replication.

C. glabrata strains (Table 1) were cultured on either YPD, containing 1% Bacto yeast extract (Difco Laboratories, Detroit, MI), 2% Bacto peptone (Difco Laboratories), and 2% glucose (Sigma, St. Louis, MO), or MIN, containing 0.67% yeast nitrogen base plus 2% glucose without amino acids (Difco Laboratories). YEPG agar was used for drug sensitivity assay and petite growth phenotype, containing 1% Bacto yeast extract (Difco Laboratories), 2% Bacto peptone (Difco Laboratories), 3% glycerol (Invitrogen), 1% ethanol (Warner-Graham Inc., Cockeysville, MD), and 2% agar (Difco Laboratories).

The two pairs of clinical isolates were selected because each pair came from a patient receiving fluconazole; the strains acquired increased fluconazole resistance during therapy, while the karyotype remained unchanged (2).

The Cg*pdr1* mutant, CgTn173S, was generated by transposon mutagenesis using the custom transposon Tn*5*<Cm URA3>, as described by Tsai et al. (36). The *ura3* mutants were selected on a MIN plus uracil agar plate containing 0.1% 5-fluoroorotic acid (FOA) (Lancaster, Pelham, NH).

Candida glabrata petite mutants were obtained from cultures grown on a YPD agar plate containing 40 μg/ml ethidium bromide for 6 days at 30°C. The petite phenotype was confirmed by inability to grow on YEPG agar.

A haploid *S. cerevisiae pdr1* mutant, 4381, and its parental strain, BY4741, were obtained from Open Biosystems (Huntsville, AL) (41).

Identification of the Tn-inserted gene. The plasmid pTn173S (Table 2) was rescued from the Cg*pdr1* mutant, CgTn173S. Briefly, the Tn-inserted genomic DNA of CgTn173S was digested with BglII, self ligated, and transformed into *E. coli* EC100D *pir*$^+$. The rescued plasmid pTn173S was sequenced using the Tn primers SqFP and SqRP (Epicenter) to obtain the sequence information of the Tn insertion site. Nucleotide sequences obtained were then used for BlastX searches against the protein database to identify homologs. The plasmid pTn173S was found to contain sequences homologous to *S. cerevisiae PDR1*. The full nucleotide sequence of the *C. glabrata PDR1* homolog (Cg*PDR1*) was obtained from the Pasteur Institute genomic database (Génolevures Consortium; *http://cbi.labri.fr/Genolevures/*). We also sequenced Cg*PDR1* from two pairs of clinical isolates.

Cg*PDR1* disruption. To confirm the association of Cg*pdr1* mutations and increases in fluconazole sensitivity, the rescued plasmid, pTn173S, was lin-

TABLE 2. Plasmids used in this study

Strain	Genotype and/or description	Reference or source
pCC1FOS	Copy control fosmid vector	Epicentre
pCC1URA3	Copy control fosmid vector	36
pCgACU	*C. glabrata* centromere vector	20
pH 392	*S. cerevisiae* centromere overexpression vector	H. Edskes[a]
pTn173S	Plasmid rescued from CgTn173S Cg*pdr1*::Tn*5*<Cm URA3>	This study
P16C3	Cg13928 Cg*PDR1* genomic fosmid clone	This study
P2F5	Cg4672 Cg*PDR1* genomic fosmid clone	This study
$pCgPDR1_{38a}$	38a Cg*PDR1* genomic clone in pBluscript SK−	This study
pCgPDR1	$ADH1_p$-Cg$PDR1_{38a}$-$ADH1_T$ in pCgACU[b]	This study
pHCgPDR1	$ADH1_p$-Cg$PDR1_{38a}$-$ADH1_T$ in pH 392[b]	This study
$pCgPDR1_{12581}$	Cg*PDR1* ORF of Cg12581 in pCR-Blunt II-TOPO	This study
$pCgPDR1_{13928}$	Cg13928 Cg*PDR1* gene in pCgACU	This study
$pCgPDR1_{1660}$	Cg*PDR1* ORF of Cg1660 in pCR-Blunt II-TOPO	This study
$pCgPDR1_{4672}$	Cg4672 Cg*PDR1* gene in pCgACU	This study

[a] A kind gift of Herman Edskes, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD.
[b] *P*, promoter; *T*, terminator.

earized with BglII and transformed into 84u (*ura3* mutant of the wild-type strain NCCLS84) to construct a Cg*pdr1* disruptant via a double-crossover homologous recombination. The disruption of Cg*PDR1* was confirmed by Southern blot analysis of the BglII-digested genomic DNA. The purified 2.9-kb Tn probe detected a single 4.2-kb signal in the putative Cg*pdr1* mutant CgB4, while there was no signal in the parental strain NCCLS84 (Fig. 1B, left panel). The 1.3-kb Cg*PDR1* probe detected the 1.3-kb signal in NCCLS84 but only hybridized to the 4.2-kb DNA in CgB4 (Fig. 1B, right panel). Cg173C, a Cg*PDR1*-complemented strain, was obtained by introducing the 8-kb KpnI genomic DNA containing the Cg*PDR1* gene of Cg13928 into the Cg*pdr1* locus of the mutant, CgB4, via a targeted gene replacement (Fig. 1).

Drug sensitivity assay. Susceptibilities to fluconazole and voriconazole (both courtesy of Pfizer, Sandwich, United Kingdom) and itraconazole (Janssen Pharmaceuticals, Titusville, NJ) were tested using the NCCLS microtiter test using 80% growth reduction (MIC_{80}) as the MIC and modified by addition of 2% glucose to the RPMI media and incubation at 37°C for 48 h (26). Disk diffusion susceptibility to cerulenin (Sigma), chloramphenicol (Sigma), cycloheximide (Sigma), and rhodamine 6G (R6G; Sigma) was tested by adding 10 μl of drug solution to 6-mm paper disks (Becton Dickinson, Sparks, MD), and we placed the disks on the agar plate seeded with 2×10^5 cells. The plates were photographed after 2 days of incubation at 30°C.

Complementation using Cg*PDR1* from different clinical isolates. A fosmid genomic library of Cg13928, a fluconazole-resistant clinical isolate, was constructed in copy control fosmid vector pCC1URA3 as described in Tsai et al. (36). The average insert size of the genomic library was about 40 kb. The Cg4672 fosmid genomic library was constructed similarly, except it was in a different copy control fosmid vector, pCC1FOS (Epicenter). For functional complementation, the fosmid genomic clones P16C3 (Cg13928) and P2F5 (Cg4672), carrying the full-length Cg*PDR1* gene, were identified via PCR screening of the fosmid genomic library using the primer set CgPDR2S (5′-TATCCTAAGTATGGACAACG-3′) and CgPDR4AS (5′-GATTCCTTAAGCCCGATAAG-3′) with the following parameters: 95°C for 2 min; 35 cycles of 95°C for 30 s, 53°C for 30 s, and 72°C for 2 min; with extension on the last cycle at 72°C for 10 min.

To complement the Tn-disrupted Cg*pdr1* in CgB4, P16C3 was digested with KpnI, and the 8-kb KpnI fragment containing the Cg*PDR1* ORF, 2.5 kb of the 5′ upstream region, and 1.9 kb of the 3′ downstream region was used to transform CgB4. Transformants were plated on MIN plus uracil agar plates containing 100 μg/ml fluconazole and incubated at 30°C for 3 days; the transformants were then replica plated onto MIN plus uracil agar plates containing 0.1% FOA. FOA-resistant transformants were obtained and analyzed by PCR using primers CgPDR2S and CgPDR4AS with the above conditions. Southern hybridization and DNA sequence analysis confirmed that the Tn-inserted Cg*pdr1* was replaced with the intact Cg*PDR1* gene of P16C3, including the 5′ upstream region via a targeted gene replacement in the complemented strain, Cg173C (Fig. 1). Absence of a tandem repeat was also confirmed by Southern blot analysis (data not shown).

The Cg*PDR1* ORF of Cg12581 and Cg1660 was obtained by PCR using *Pfu* Ultra DNA polymerase (Stratagene) with primers CgPDR8S (5′-GGTGGAGCTCTTTAGCTACGTTATTGAG) and CgPDR5AS (5′-GGTTACACCACTACTAGTTG). The 3.6-kb PCR product was cloned into pCR-BluntII-TOPO (Invitrogen) to yield the plasmids pCg$PDR1_{12581}$ and pCg$PDR1_{1660}$, which were sequenced and confirmed. The plasmid pCg$PDR1_{13928}$, containing the Cg*PDR1* gene of Cg13928, was obtained by subcloning the 8-kb KpnI DNA fragment from the fosmid clone P16C3 into pCgACU (20). Similarly, the plasmid pCg$PDR1_{4672}$, containing the Cg*PDR1* gene of Cg4672, was obtained by subcloning the 8-kb KpnI DNA fragment from the fosmid clone P2F5 into pCgACU. The 2.9-kb HindIII fragments of the susceptible (Cg12581 and Cg1660) and resistant (Cg13928 and Cg4672) clinical isolates were purified from HindIII-digested $pCgPDR1_{12581}$, $pCgPDR1_{1660}$, $pCgPDR1_{13928}$, and $pCgPDR1_{4672}$; the 2.9-kb HindIII fragments containing the partial Cg*PDR1* ORF were then used to transform the Cg*pdr1* mutant CgB4, which is highly susceptible to fluconazole. Putative transformants were obtained based on the restoration of wild-type fluconazole susceptibility at 50 μg/ml and loss of *URA3*. Briefly, the transformants were plated on a MIN plus uracil agar plate containing 50 μg/ml fluconazole and incubated at 30°C for 3 days; they were then replica plated onto a MIN plus uracil agar plate containing 0.1% FOA. Restoration of Cg*PDR1* could only result from the targeted integration of the partial Cg*PDR1* into the mutated Cg*pdr1* locus. An ectopic integration of the partial Cg*PDR1* could not result in an intact Cg*PDR1*. Two independent PCR amplifications using *Pfu* Ultra DNA polymerase and DNA sequencing confirmed the targeted replacement of Cg*PDR1* ORF in the transformants.

Cg*PDR1* overexpression. The promoter of *S. cerevisiae ADH1* was used for Cg*PDR1* overexpression in both *S. cerevisiae* and *C. glabrata*. The Cg*PDR1* ORF, along with the 24-bp 5′ upstream region and 335-bp 3′ downstream region, was obtained by PCR using *Pfu* Turbo DNA polymerase with primers CgPDR5AS (5′-GGTTACACCACTACTAGTTG) and CgPDR8S (5′-GGTGGAGCTCTTT

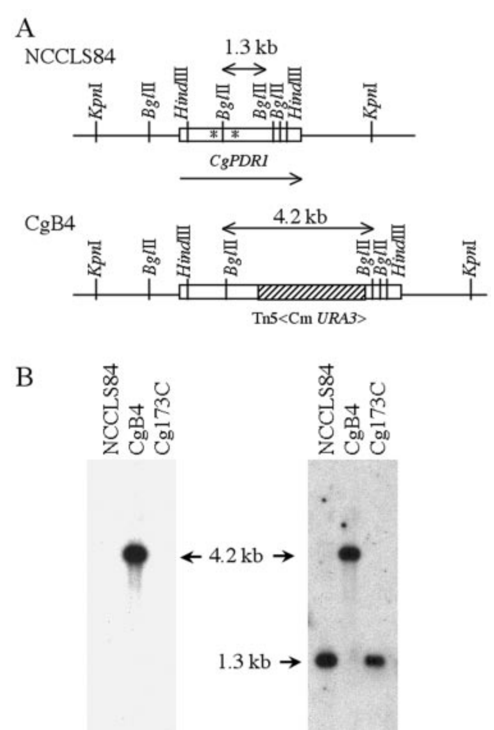

FIG. 1. Southern hybridization analysis confirmed the disruption and targeted replacement of *PDR1* in *C. glabrata*. (A) Restriction enzyme map. (B) Southern blot analysis. Total DNA was digested with BglII. The membrane was hybridized with the 2.9-kb Tn*5*<Cm URA3> (left panel) or 1.3-kb BglII Cg*PDR1* (right panel) probe. Lanes: 1, strain NCCLS84, *C. glabrata* laboratory wild-type strain; 2, strain CgB4, Cg*pdr1* mutant; 3, strain Cg173C, CgB4 complemented with Cg*PDR1* of clinical fluconazole-resistant isolate Cg13928. An asterisk indicates the codon mutations in the clinical fluconazole-resistant isolates, W297S in Cg4672 and F575L in Cg13928.

AGCTACGTTATTGAG). The underlined SacI site was created for cloning purposes. The 3.7-kb PCR product was cloned into pCRScript Amp (Stratagene). DNA sequencing confirmed the sequence accuracy of the 0.5-kb insert at both ends. The 3.7-kb Cg*PDR1* insert was excised with NotI (blunt-ended with T4 DNA polymerase)-SacI and was subcloned into the HindIII (also blunt-ended)-SacI site of the yeast centromere overexpression vector pH392. To avoid mutations introduced by PCR in the nonsequenced coding region, the 2.9-kb HindIII coding region was replaced with the 2.9-kb HindIII fragment from the Cg*PDR1* genomic clone pCgPDR1$_{38a}$ to give the construct pHCgPDR1. The plasmid pCgPDR1$_{38a}$ was obtained from a custom genomic library of *C. glabrata* isolate 38a (Stratagene) (25) by hybridization screening using the Cg*PDR1*-specific probe. For overexpression in *C. glabrata*, the overexpression construct pCgPDR1 was obtained by excising the Cg*PDR1* overexpression cassette from pHCgPDR1 with BglI plus SpeI and subcloning into the SmaI site of the *C. glabrata* centromere vector pCgACU.

Rhodamine 6G accumulation analysis. Accumulation of rhodamine 6G (R6G) (Sigma) was measured by flow cytometry in a FACSCalibur fluorescence-activated cell cytometer (Becton Dickinson, San Jose, CA) (14, 24). Overnight cultures grown at 30°C in MIN were diluted to an optical density at 600 nm of 1 with MIN and shaken an additional 2 h at 30°C. Rhodamine 6G (R6G) was added to give a final concentration of 0.2 μg/ml and was shaken an additional 4 h at 30°C. After incubation, 10 μl of the culture was transferred to 0.9 ml of ice-cold phosphate-buffered saline (PBS) at pH 7.0 and incubated on ice for 5 min before fluorescent-activated cell sorter (FACS) analysis. A total of 20,000 cells were scanned using the 488-nm laser and FL-2 filter. Cultures without R6G were also analyzed and served as unstained controls. Data were analyzed with CellQuest (Becton Dickinson) and FlowJo programs (Tree Star Inc., San Carols, CA). Fluorescence was expressed as geometric mean values.

Effect of drugs on Cg*CDR1* expression. A *C. glabrata* overnight culture was refreshed in MIN broth and incubated for 3 h in a 30°C shaker; it was subsequently incubated for an additional 2 h after addition of 1 μg/ml of oligomycin (Sigma), 1 μg/ml of cycloheximide, or 100 μg/ml of rhodamaine 6G prior to extraction of RNA. *C. glabrata* RNA was isolated from log-phase cultures with a FastRNA Pro-Red kit (QBIOgene, Carlsbad, CA).

Techniques and reagents. *C. glabrata* genomic DNA was isolated from overnight cultures using the MasterPure Yeast Purification kit (Epicenter). Purified DNA fragments were recovered using a Geneclean Spin kit (QBIOgene). Hybond-N nylon membranes (Amersham, Arlington Heights, IL) were used for Southern and Northern blot analyses. DNA probes were labeled with [α-^{32}P]dCTP or [α-^{32}P]dATP (Amersham) using the Prime-It II kit. DNA cloning and hybridization analyses were done according to the standard protocols (32). DNA sequencing was done using a DNA sequencing kit with a dRhodamine terminator (Applied Biosystems, Foster City, CA) and an ABI automatic DNA Sequencing system (Perkin-Elmer, Foster City, CA). For sequencing of PCR products, *Pfu* Ultra DNA polymerase (Stratagene) was used for PCR amplification to minimize the rate of PCR-introduced mutation. The PCR products were cleaned with a Strataprep PCR purification kit (Stratagene) and used as templates for DNA sequencing.

Nucleotide sequence accession numbers. The sequence data determined in the course of this work have been submitted to the GenBank under accession numbers DQ174090 (Cg12581), DQ174091 (Cg13928), DQ174092 (Cg1660), and DQ174093 (Cg4672).

RESULTS

Cg*PDR1* affected azole susceptibility and complemented the *S. cerevisiae pdr1* mutant. The Cg*pdr1* mutant CgTn173S obtained via transposon (Tn) mutagenesis showed increased sensitivity to fluconazole compared to its parental clinical isolate, Cg1660 (Table 3). Southern blot analysis using the transposon probe showed that there was a single transposon insertion in CgTn173S (data not shown). The inserted Tn along with the flanking genomic DNA from CgTn173S was recovered and sequenced to characterize the gene disrupted by the transposon. BlastX search of GenBank with the obtained sequences indicated that the sequences had the greatest homology with *S. cerevisiae PDR1*, which encodes a zinc finger transcriptional factor of 1,068 amino acids. Protein sequence comparison using the GCG Bestfit program showed that the putative protein had 40% identity and 52% similarity with Pdr1p versus 35% identity and 47% similarity with Pdr3p. Therefore, the gene was designated Cg*PDR1*, which encodes a putative protein of 1,107 amino acids. The transposon had inserted at the codon of amino acid 748.

TABLE 3. Azole susceptibility of *C. glabrata* strains

Strain	MIC$_{80}$ (μg/ml) of:		
	Fluconazole	Voriconazole	Itraconazole
38a	32	ND[b]	ND
Cg12581	16	ND	ND
Cg13928	128	ND	ND
Cg1660	32–64	ND	ND
Cg4672	256	ND	ND
CgTn173S	4	0.0625	0.5
NCCLS84	64	1	4–8
CgB4	4–8	0.125	0.5
CgB4u/pCgACU	4–8	0.0625	0.5
CgB4u/pCgPDR1	512	8	>32
Cg173C	512	4–8	>32
NCCLS84	64[a]	ND	ND
CgB4	16[a]	ND	ND
CgB4Ca$_{12581}$	32–64[a]	ND	ND
CgB4Cb$_{12581}$	32–64[a]	ND	ND
CgB4Ca$_{13928}$	256[a]	ND	ND
CgB4Cb$_{13928}$	256–512[a]	ND	ND
CgB4Ca$_{1660}$	64[a]	ND	ND
CgB4Cb$_{1660}$	32–64[a]	ND	ND
CgB4Ca$_{4672}$	512[a]	ND	ND
CgB4Cb$_{4672}$	>512[a]	ND	ND

[a] RPMI medium supplemented with 8 μg/ml uracil.
[b] ND, not determined.

Cg*PDR1* was disrupted in a laboratory strain to further confirm the association of Cg*pdr1* mutations and increased fluconazole susceptibility. The Cg*pdr1* disruptant CgB4 had 8- to 16-fold increased susceptibility to the azoles fluconazole, voriconazole, and itraconazole compared to its parental strain, NCCLS84 (Fig. 1 and Table 3). Complementation of the Cg*pdr1* disruption by overexpressing Cg*PDR1* with the *S. cerevisiae ADH1* promoter conferred drug resistance. CgB4u, the *ura3* mutant of CgB4, transformed with pCgACU, the shuttle vector, was used as a negative control and did not reduce azole susceptibility. In contrast, CgB4u/pCgPDR1, the complemented strain, showed an 8- to 16-fold increased resistance to the three azoles compared to NCCLS84 (Table 3). Finally, Cg*PDR1* was shown to complement a commercially obtained *S. cerevisiae pdr1* mutant (Fig. 2). Disk diffusion assays showed that *S. cerevisiae pdr1* mutant 4381 had increased sensitivities to fluconazole and rhodamine 6G compared to its wild-type strain, BY4741 (Fig. 2). Importantly, the Cg*PDR1* overexpression construct, pHCgPDR1, reduced the sensitivities of *pdr1* mutant 4381 to fluconazole and rhodamine 6G (Fig. 2).

Cg*PDR1* regulated the gene expression of ABC transporters and efflux of rhodamine 6G. Northern blot analysis with the Cg*PDR1* probe showed that the Cg*PDR1* transcript was estimated to be 3.5 kb in size and was present at a low level in the wild-type strain NCCLS84 (Fig. 3). In contrast, the native Cg*PDR1* transcript was absent in the Cg*pdr1* mutant CgB4, which had slightly increased expression of a truncated Cg*pdr1* transcript 2.2 kb in size. As *PDR1* regulates the gene expression of ABC transporters in *S. cerevisiae*, the effect of Cg*PDR1* on the gene expression of the two ABC transporters, CgCdr1p

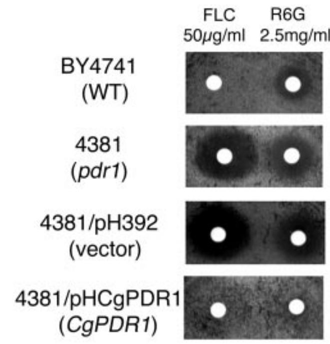

FIG. 2. Overexpression of Cg*PDR1* in the *pdr1* mutant of *S. cerevisiae* reduced its sensitivities to fluconazole and rhodamine 6G. A disk diffusion assay was used to determine the drug sensitivities of *S. cerevisiae* strains. Strains: BY4741, wild type; 4381, *pdr1* mutant; 4381/pH392, *pdr1* mutant carrying the vector pH392; 4381/pHCgPDR1, *pdr1* mutant carrying the Cg*PDR1* overexpression cassette $ADH1_p$-Cg$PDR1$-$ADH1_T$. FLC, fluconazole; R6G, rhodamine 6G. Plates were photographed after 2 days of incubation at 30°C. WT, wild type.

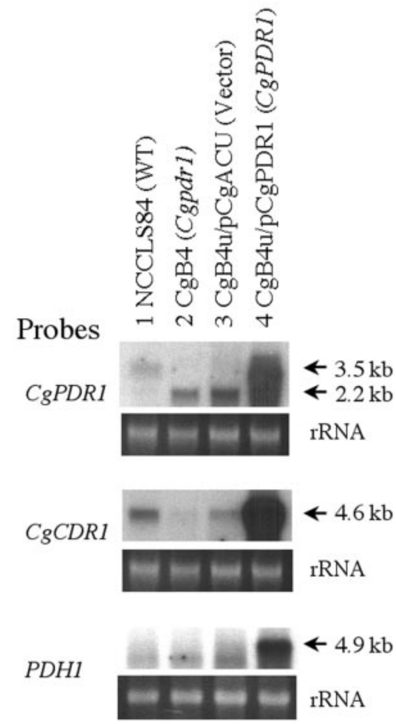

FIG. 3. Disruption or overexpression of Cg*PDR1* altered the expression of Cg*CDR1* and *PDH1*. Northern hybridization analyses were used to determine the expression of Cg*PDR1*, Cg*CDR1*, and *PDH1*. Ten micrograms of total RNA was used for Northern blot analysis. The membrane was hybridized with the following probe: 2.3-kb BglII Cg*PDR1* DNA (top panel), 1.1-kb NotI-BamHI Cg*CDR1* DNA from pCRScript-CDR1 (14) (middle panel), or 3.6-kb *PDH1* DNA from pClaI (25) (bottom panel). Sizes of putative transcripts are indicated in kilobases on the right. The rRNA stained with ethidium bromide was used as the loading control. Lanes: 1, strain NCCLS84, wild type; 2, strain CgB4, C*gpdr1* mutant; 3, strain CgB4/pCgACU, C*gpdr1* mutant carrying the vector pCgACU; 4, strain CgB4/pCgPDR1, C*gpdr1* mutant overexpressing Cg*PDR1*. WT, wild type.

and Pdh1p, was analyzed. The expression of Cg*CDR1* in CgB4 was reduced compared to that in NCCLS84, while the *PDH1* transcript was not detected in both NCCLS84 and CgB4 due to its low basal expression. In contrast, overexpression of Cg*PDR1* in CgB4u (strain CgB4u/pCgPDR1) resulted not only in abundant expression of Cg*PDR1* but also a dramatic increase in the expression of both ABC transporter genes, Cg*CDR1* and *PDH1*. A putative pleiotropic drug resistance responsive element (PDRE), TTCCGTGGAA, is present in the upstream region of both Cg*CDR1* (position −1201 to −1192 from the translation start site ATG) and *PDH1* (position −560 to −551 from the translation start site ATG). Rhodamine 6G, which is effluxed by these two ABC transporters, was used to assess the effect of regulation by Cg*PDR1*. FACS analyses showed that the strain CgB4u/pCgACU (vector only) had greater rhodamine 6G accumulation than NCCLS84, and rhodamine 6G accumulation was greatly reduced in the strain CgB4u/pCgPDR1 (Table 4). Therefore, we conclude that Cg*PDR1* affected the expression of ABC transporter genes as well as rhodamine 6G efflux.

Cg*PDR1* mediated the drug-induced expression of Cg*CDR1*. The expression of Cg*CDR1* can be induced by drug treatment. Northern blot analyses showed that Cg*CDR1* expression was induced moderately by rhodamine 6G, and disruption of C*gpdr1* slightly reduced the rhodamine 6G-induced expression (Fig. 4). Fluconazole, on the other hand, is a weak inducer based on our Northern blot analysis (data not shown). In contrast, the Cg*CDR1* expression was induced dramatically by cycloheximide and oligomycin in NCCLS84. Northern blot analyses showed that disruption of Cg*PDR1* abolished the induced expression of Cg*CDR1* by cycloheximide and oligomycin in CgB4. Thus, Cg*PDR1* is the major regulator of the cycloheximide- and oligomycin-induced Cg*CDR1* expression.

Cg*PDR1* affected drug resistance. The C*gpdr1* mutant CgB4 exhibited increased sensitivity to rhodamine 6G, cycloheximide, chloramphenicol, and cerulenin (Fig. 5). Disk diffusion assays showed that NCCLS84 was resistant to 0.5 mg/ml of fluconazole, 2.5 mg/ml of rhodamine 6G (R6G), 20 μg/ml of cycloheximide, and 100 mg/ml of chloramphenicol, with only borderline inhibition by 250 μg/ml cerulenin (Fig. 5). In contrast to NCCLS84, in which no clear growth inhibition zones were observed, clear growth inhibition zones were observed with CgB4, indicating its increased drug sensitivities. The plasmid pCgACU alone (CgB4u/pCgACU) seemed to reduce the growth inhibition zone of CgB4u slightly in general. However, overexpression of Cg*PDR1* in CgB4u (CgB4u/pCgPDR1) completely eliminated the growth inhibition zone, which indicated restoration of drug resistance by Cg*PDR1* overexpression in CgB4u. As Cg*PDR1* regulated the expression of ABC transporter genes Cg*PDR1* and *PDH1*, the drug sensitivity of a

TABLE 4. Rhodamine 6G accumulation in *C. glabrata* strains

Strain	Geometric mean fluorescence (in arbitrary units)	
	Without R6G	With R6G
NCCLS84	2.6	9.6
CgB4	2.2	23.1
CgB4u/pCgACU	2.0	25.4
CgB4u/pCgPDR1	2.0	3.1
Cg173C	1.9	2.7

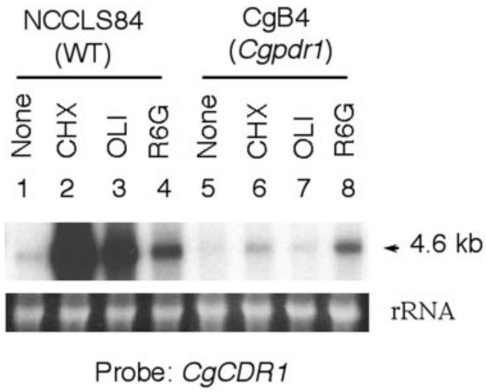

FIG. 4. Cycloheximide- and oligomycin-induced Cg*CDR1* expression depended on Cg*PDR1*. Ten micrograms of total RNA was used for the Northern hybridization analysis to determine the level of drug-induced Cg*CDR1* expression. The membrane was hybridized with the 1.1-kb NotI-BamHI Cg*CDR1* DNA probe. The sizes of putative transcripts are indicated in kilobases. The rRNA stained with ethidium bromide was used as the loading control. Lanes: 1 to 4, NCCLS84 (wild type); 5 to 8, CgB4 (Cg*pdr1* mutant). CHX, cycloheximide; OLI, oligomycin; R6G, rhodamine 6G. WT, wild type.

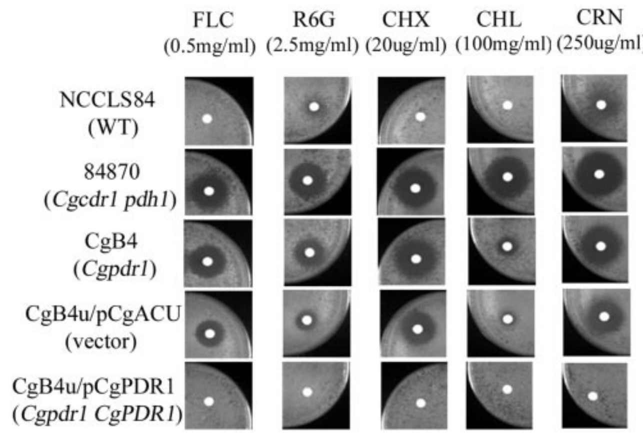

FIG. 5. Altered drug sensitivity in *C. glabrata* caused by the disruption or overexpression of Cg*PDR1*. Disk diffusion assay was used to determine the drug sensitivity of *C. glabrata*. Strains: NCCLS84, wild type; 84870, Cg*cdr1* and *pdh1* double disruptant; CgB4, Cg*pdr1* mutant; CgB4u/pCgACU, Cg*pdr1* carrying the vector pCgACU; CgB4u/pCgPDR1, Cg*pdr1* mutant overexpressing Cg*PDR1*. FLC, fluconazole; R6G, rhodamine 6G; CHX, cycloheximide; CHL, chloramphenicol; CRN, cerulenin. Plates were photographed after 2 days of incubation at 30°C. WT, wild type.

transporter disruptant was also analyzed. The Cg*cdr1* and *pdh1* double disruptant, 84870, had greater sensitivity to rhodamine 6G and chloramphenicol than CgB4. It is possible that basal expression of these transporters was not completely absent when transcriptional regulation by Cg*PDR1* was lost.

Increased Cg*PDR1* and Cg*CDR1* expression in clinical isolates. As overexpression of Cg*PDR1* in *C. glabrata* contributed to drug resistance in the laboratory setting, it would be of interest to determine whether it also contributes to drug resistance developed in a clinical setting. Northern blot analyses were done with two pairs of clinical azole-sensitive and -resistant isolates, which had four- to eightfold differences in fluconazole MIC_{80}s. Both resistant isolates Cg4672 and Cg13928 had higher expression of Cg*PDR1* and Cg*CDR1* than their paired fluconazole-sensitive strains, Cg1660 and Cg12581 (Fig. 6).

Upregulated PDR gene expression and increased fluconazole resistance in Cg13928 linked to its Cg*PDR1* locus. Cg*PDR1* of Cg13928, along with its promoter region, was introduced into the disrupted Cg*pdr1* locus of CgB4 via an integrative transformation to complement the Cg*pdr1* mutation. The complemented strain, Cg173C, showed an 8- to 16-fold increase in the resistance to three azoles compared to that of NCCLS84 (Table 3 and Fig. 1). Northern blot analysis showed that the expression of Cg*PDR1* and Cg*CDR1* was also upregulated in Cg173C (data not shown). In agreement, FACS analysis showed that the rhodamine 6G accumulation was greatly reduced in Cg173C compared to NCCLS84 and CgB4 (Table 4). Together, our data indicated that Cg*PDR1* and its promoter region were responsible for the fluconazole resistance in the clinical fluconazole-resistant isolate Cg13928.

F575L and W297S codon substitutions in Cg*PDR1* increased Cg*PDR1* expression and fluconazole resistance of clinical isolates. It was unclear whether the azole resistance and increased Cg*PDR1* expression in the resistant isolates of each pair was due to the promoter or regulatory elements in the flanking regions or to mutations in the Cg*PDR1* ORF. Therefore, Cg*PDR1* from the two pairs of clinical isolates was sequenced. Analyses of the Cg*PDR1* coding region as well as the 2.5-kb upstream regions revealed no differences in the flanking regions, and the Cg*PDR1* ORF differed between the susceptible and resistant isolates at a single codon in each pair.

The Cg*PDR1* sequence of Cg13928 contained a point mutation at codon 575 (TTC to CTC) compared to the Cg*PDR1* sequences of Cg12581. F575L is located in the fungus-specific transcription factor domain of CgPdr1p, according to the homology of CgPdr1p with *S. cerevisiae* Pdr1p. The Cg*PDR1* sequence of Cg4672 contained a single mutation at codon 297 (TGG to TCG) compared to the Cg*PDR1* sequences of Cg1660. W297S is located in the inhibition domain of CgPdr1p

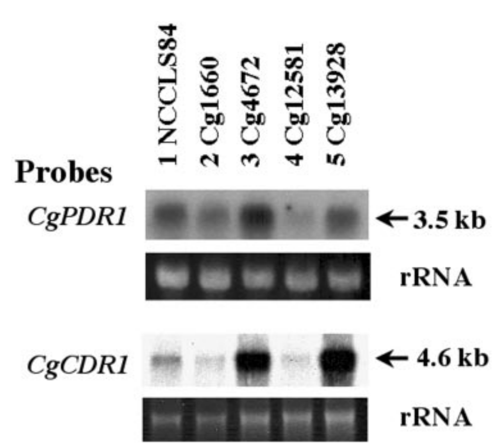

FIG. 6. Clinical fluconazole-resistant isolates overexpressed Cg*PDR1* and Cg*CDR1*. Two pairs of clinical isolates, Cg1660 versus Cg4672 and Cg12581 versus Cg13928, were analyzed by Northern blot hybridization along with NCCLS84. Probes used for each Northern blot are labeled on the left, and the estimated sizes of hybridized transcripts in kilobases are labeled on the right. The rRNA stained with ethidium bromide was used as the loading control. Probes: top, 2.3-kb *Bgl*II Cg*PDR1* DNA; bottom, 1.1-kb NotI-BamHI Cg*CDR1* DNA.

TABLE 5. Azole susceptibility of *C. glabrata* and petite mutants

Strain	MIC_{80} (μg/ml) of:		
	Fluconazole	Voriconazole	Itraconazole
NCCLS84	64	1	4
84p1	>512	16	>32
84p2	512	>32	>32
84p3	512	16	16
CgB4	8	0.125	0.5
CgB4p3	8	0.125	1–2
CgB4p4	8	0.5	2
CgB4p5	8	0.5	2

in Cg4672, according to the homology with *S. cerevisiae* Pdr1p. The data indicated that even though the two clinical fluconazole-resistant isolates had upregulated Cg*PDR1* expression, they harbored different mutations.

Direct correlation of the mutations with upregulated Cg*PDR1* expression as well as increased fluconazole resistance was further investigated by gene substitution and Northern blot analyses. Both mutated codons, W297S and P595L, were within the 2.9-kb HindIII region of the Cg*PDR1* ORF. The 2.9-kb HindIII fragments containing the partial Cg*PDR1* ORF (amino acids 30 to 974) from Cg12581, Cg13928, Cg1660, and Cg4672 were introduced into the mutated Cg*pdr1* locus of CgB4 independently by integrative transformation. Two transformants, labeled "CgB4Ca" and "CgB4Cb," were selected from each integrative transformation for sequence analysis and fluconazole susceptibility assay. The presence of the correct nucleotide sequences in the transformants was verified by DNA sequencing. In Table 3, the fluconazole susceptibilities of the transformants can be seen to correspond to those of the clinical isolates from which the Cg*PDR1* gene was obtained. In agreement, Northern blot analyses showed that the transformants carrying the Cg13928 or Cg4672 Cg*PDR1* had increased expression of Cg*PDR1* and Cg*CDR1* compared to the transformants carrying the Cg12581 or Cg1660 Cg*PDR1* as well as the wild type (Fig. 7). Thus, we concluded that the W297S and F575L single-amino-acid substitutions were the cause of upregulated Cg*PDR1* and Cg*CDR1* expression as well as the increased fluconazole resistance in the clinical fluconazole-resistant isolates Cg4672 and Cg13928, respectively.

Cg*PDR1* is required for fluconazole resistance in petite mutants. Similar to *S. cerevisiae*, petite mutants of *C. glabrata* also exhibited increased fluconazole resistance. *PDR3*, not *PDR1*, was overexpressed in the *S. cerevisiae* petite mutants and was shown to be essential for the upregulated expression of *PDR5* in the petite mutants. To date, only the *PDR1* homologue has been annotated in the genome database of *C. glabrata.* We postulated that Cg*PDR1* might be the transcriptional factor responsible for azole resistance in the *C. glabrata* petite mutants. Therefore, three petite mutants from both the wild-type strain, NCCLS84, as well as the Cg*pdr1* mutant, CgB4, were analyzed. MIC_{80} assays showed that the petite mutants of NCCLS84 exhibited 8-fold or greater resistance to fluconazole, 16-fold or greater resistance to voriconazole, and 4-fold or greater resistance to itraconazole compared to its parental strain NCCLS84 (Table 5). In addition, Northern blot analyses showed the increased expression of Cg*PDR1*, the transcriptional factor, and its transcriptional target, Cg*CDR1*, the transporter gene in the three petite mutants of NCCLS84 (Fig. 8). In contrast, three petite mutants of CgB4 exhibited unaltered susceptibility to fluconazole, one- to fourfold increased resistance to voriconazole, and two- to fourfold increased resistance to itraconazole compared to its parental strain, CgB4 (Table 5). Northern blot analysis showed that the expression of the truncated Cg*pdr1* gene was slightly increased in the three petite mutants of CgB4 compared to their parental strain, CgB4, but there was no obvious change in the Cg*CDR1* expression of the three CgB4 petite mutants. We conclude that the functioning Cg*PDR1* was essential for mediating fluconazole resistance in the petite mutants of *C. glabrata*.

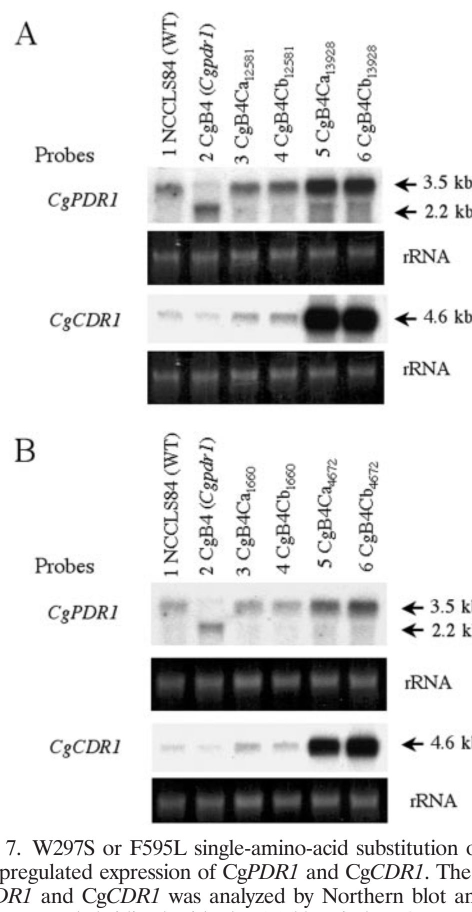

FIG. 7. W297S or F595L single-amino-acid substitution of CgPdr1p led to upregulated expression of Cg*PDR1* and Cg*CDR1*. The expression of Cg*PDR1* and Cg*CDR1* was analyzed by Northern blot analysis. The membrane was hybridized with the 2.9-kb HindIII Cg*PDR1* DNA or 1.1-kb NotI-BamHI Cg*CDR1* DNA probe. The rRNA stained with ethidium bromide was used as the loading control. All complementations were done with integrative transformation. (A) Lanes: 1, strain NCCLS84, wild type; 2, strain CgB4, Cg*pdr1* mutant; 3 and 4, strain $CgB4Ca_{12581}$ and strain $CgB4Cb_{12581}$, CgB4 complemented by Cg*PDR1* from Cg12581; 5 and 6, strain $CgB4Ca_{13928}$ and strain $CgB4Cb_{13928}$, CgB4 complemented by Cg*PDR1* from Cg13928. (B) Lanes: 1, strain NCCLS84, wild type; 2, strain CgB4, Cg*pdr1* mutant; 3 and 4, strain $CgB4Ca_{1660}$ and strain $CgB4Cb_{1660}$, CgB4 complemented by Cg*PDR1* from Cg1660; 5 and 6, strain $CgB4Ca_{4672}$ and strain $CgB4Cb_{4672}$, CgB4 complemented by Cg*PDR1* from Cg4672. WT, wild type.

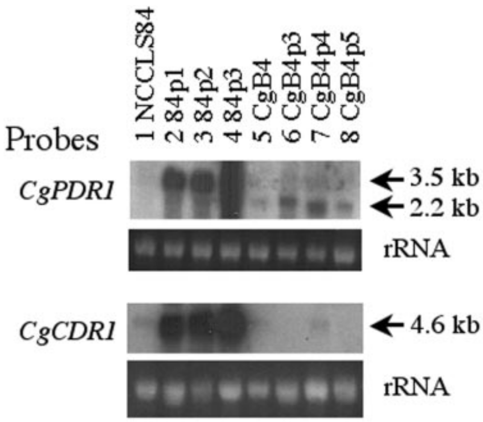

FIG. 8. Cg*PDR1* is required for upregulated expression of PDR genes caused by mitochondrial dysfunction. For Northern blot analysis, the membrane was hybridized with the 2.3-kb BglII Cg*PDR1* DNA (top) or 2.3-kb BamHI-NotI Cg*CDR1* (bottom) probe. The rRNA stained with ethidium bromide was used as the loading control. Lanes: 1, strain NCCLS84, wild type; 2 and 3, petite mutant strains derived from NCCLS84; 5, strain CgB4, Cg*pdr1* mutant; 6 to 8, petite mutant strains derived from CgB4.

DISCUSSION

Increased expression of multidrug transporter genes has been shown in both laboratory and clinical azole-resistant isolates of *Candida* species (25, 28, 31, 34, 40). In *S. cerevisiae*, gain-of-function mutations in either *PDR1* or *PDR3* led to increased expression of the ABC transporter *PDR5*. However, Cg*PDR1* mutation and upregulation has only been identified in one laboratory azole-resistant *C. glabrata* isolate to date, with no evidence that the mutation caused the upregulation (38). Here, for the first time, we showed that upregulation of the Cg*PDR1* expression indeed occurred in the clinical azole-resistant isolates and petite mutants, which contributed to the drug resistance by upregulating the expression of Cg*CDR1*.

We identified that only a single-amino-acid substitution in CgPdr1p, W297S in Cg4672 and F575L in Cg13928, could lead to increased Cg*PDR1* expression and azole resistance. The putative functional domains that W297S and F575L mutations located were determined based on the homology of CgPdr1p with *S. cerevisiae* Pdr1p. Amino acid 297 is located near the region corresponding to the inhibitory domain of *S. cerevisiae* Pdr1p. In *S. cerevisiae*, several gain-of-function mutations have been observed in the domain, e.g., K302Q (*pdr1-6*) and M308I (*pdr1-2*) (6). In contrast, the altered F595L codon is located in the region corresponding to the fungus-specific transcription factor domain, which has not been reported to associate with fluconazole resistance. In *S. cerevisiae*, gain-of-function mutations in *PDR1* are also located in the activation domain (amino acids 879 to 1036) (21, 42). The amino acid substitution identified by Vermitsky and Edlind as possibly increasing transcriptional activity, P927L, was located in the carboxy-terminal domain of CgPdr1p, not the fungus-specific domain (38). It would be of interest to further investigate the importance of the codon 575 in the function of CgPdr1p.

Both W297S and F575L mutations have resulted in the upregulated expression of Cg*PDR1*. One explanation is that the mutations altered CgPdr1p conformation and CgPdr1p became hyperactive, which may directly or indirectly affect the expression of Cg*PDR1*. The possibility of autoregulation will be investigated as a putative PDRE, "TTCCGTGGAA," is observed at the 5′ upstream region of Cg*PDR1* and located at position −558 to −549 (upstream from the translation start site ATG). The other possibility is that the mutations increase the Cg*PDR1* mRNA half-life and thus resulted in the accumulation of Cg*PDR1* mRNA. However, as the two mutations are located at different functional domains, the upregulation of Cg*PDR1* expression likely involved different mechanisms. As shown in Fig. 8, mitochondrial dysfunction increased the expression of Cg*PDR1*. In addition, Northern blot analysis showed that cycloheximide also induced the expression of Cg*PDR1* (data not shown). These facts suggested the possibility that multiple pathways regulate Cg*PDR1* expression.

Our studies showed that Cg*PDR1* is essential in the azole resistance caused by mitochondrial dysfunction in *C. glabrata* petite mutants. In contrast, *PDR3* is the transcriptional factor essential for drug resistance in the petite mutants and *PDR1* is not involved in the resistance due to mitochondrial dysfunction *S. cerevisiae* (12, 44). In that species, *PDR3* was shown to be under positive autoregulation by Pdr3p (8), and retrograde signaling pathways have been shown to upregulate *PDR3* expression in the petite mutants of *S. cerevisiae* (10, 12). Our observation of dramatic increases of Cg*PDR1* expression in the NCCLS84 petite mutants but not in the CgB4 petite mutants suggests that Cg*PDR1* expression also may be regulated by itself in responding to mitochondrial dysfunction. However, the precise interplay mechanisms of PDR transcription factors and mitochondrial dysfunction require further exploration in both *S. cerevisiae* and *C. glabrata*.

Disruption of Cg*pdr1* led to slightly decreased rhodamine 6G-induced expression of Cg*CDR1*. In contrast, the dramatic induction of Cg*CDR1* expression by cycloheximide and oligomycin was abolished by disruption of Cg*PDR1*. These data indicated that Cg*PDR1* is the major regulator of cycloheximide- and oligomycin-induced Cg*CDR1* expression but not rhodamine 6G-induced expression.

Though the Cg*cdr1* and *pdh1* double disruptant (84870) and the Cg*pdr1* disruptant (CgB4) both had increased sensitivities to various drugs, their sensitivity profiles are not identical. The mutant 84870 had greater sensitivity to rhodamine 6G and chloramphenicol than CgB4. Even though rhodamine 6G can be effluxed by CgCdr1p, rhodamine 6G and chloramphenicol were reported to be the preferred substrates of Pdh1p (14); the difference of 84870 and CgB4 in rhodamine 6G and chloramphenicol sensitivity may be mainly due to *PDH1* expression. It is possible that disruption of Cg*PDR1* has not completely abolished the expression of one or both of these transporters.

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